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Amanita subjunquillea and its ectomycorrhizal association, reported as new for Pakistan

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Abstract—*Amanita subjunquillea* and its ectomycorrhizal association are reported for the first time from moist temperate Himalayan forests of Pakistan. The sample was studied based on morphological characters and nucleotide sequence analyses of the ITS region generated from basidiomata and ectomycorrhizal roots of *Quercus floribunda.* Our collection differs from the type in its dark orange pileus disc and pale yellow margins. Remaining morphological and molecular data are consistent with previously reported specimens. This represents the first report of *A. subjunquillea* from Pakistan.

Keywords—*Amanitaceae, Basidiomycota, Phalloideae,* ribosomal DNA, taxonomy

Introduction

Amanita Pers. (*Amanitaceae, Agaricales, Basidiomycota*) is a large genus comprising about 1000 described species that are important economically and ecologically (Bas 2000, Tulloss 2005, Yang 2005, Kirk & al. 2008, Li & al. 2015, Zhang & al. 2015, Cui & al. 2018, Yang & al. 2018). *Amanita* contains both edible and poisonous species, and some species form ectomycorrhizal associations with vascular plants, which play a key role in maintaining healthy ecosystems (Zhang & al. 2004, 2010; Thongbai & al. 2016; Cui & al. 2018; Yang & al. 2018). Poisonous *Amanita* species are classified mostly in *A.* sect. *Phalloideae* (Fr.) Konrad & Maublanc, which is characterized by the absence of striation and

lack of appendiculate pileus and the possession of attenuate lamellulae, an annulus, a stipe with a bulbous base and limbate shaped volva, and amyloid basidiospores (Corner & Bas 1962, Bas 1969, Yang 2005, Cai & al. 2016, Cui & al. 2018). *Amanita subjunquillea,* also known as the East Asian death cap, is a poisonous mushroom broadly distributed in diverse habitats in China, India, Japan, and South Korea (Bhatt & al. 2003, 2007; Yang 2005; Zhang & al. 2010; Cui & al. 2018). Symptoms of *A. subjunquillea* poisonings are gastrointestinal in nature and similar to *A. phalloides,* and ingestion can result in death (Rho & al. 2000, Kim & al. 2008).

A total of 24 species of *Amanita* have been reported from Pakistan, but it is estimated that the true number of *Amanita* from Pakistan could be above 50 (Kiran & al. 2018, Jabeen & al. 2019, Saba & al. 2019). Many collections of *Amanita* have been identified locally based on morphological features; however, such identifications are not reliable because some poisonous and edible mushrooms are morphologically similar. The Korea Forest Service reported the occurrence of twenty-three fatal mushroom poisonings in Korea during 2004–13 (Cho & al. 2015; www.forest.go.kr/). Such cases also occur every year in rural areas of Pakistan, especially in hilly areas of Swat, Khyber Pakhtunkhwa. The aim of this study was to confirm our collection of *A. subjunquillea* from Buner District as a new record for Pakistan using morphological, molecular and phylogenetic data.

Materials & methods

Sampling site

Collections were made at Bottle Green Mountains, Gokand Valley, Buner District, Khyber Pakhtunkhwa, Pakistan, at 3183 m a.s.l. The climate of Gokand is subtropical at lower elevations and temperate above. This area is covered by mixed forests of conifers (*Pinus roxburghii* Sarg., *P. wallichiana* A. B. Jacks.) and broadleaf trees (*Populus ciliata* Wall. ex Royle, *Q. floribunda* Lindl. ex A. Camus [= *"Q. dilatata"* Royle], *Q. oblongata* D. Don [= *Q. incana* Roxb.], *Zanthoxylum armatum* DC., and *Olea europaea* subsp. *cuspidata* (Wall. & G. Don) Cif. [= *O. ferruginea* Royle]). Blue pine (*P. wallichiana*) and chir pine (*P. roxburghii*) forests predominate (Khan & al. 2003).

The area was sampled during rainy seasons in July and August in 2017. Specimens were tagged and photographed on site. Morphological characters were recorded from fresh fruiting bodies in field, with colors coded according to Munsell Soil Color Charts (1975). Specimens were then dried using a fan heater and placed in small zip-lock bags for long-term preservation.

Sampling, Isolation, and morphological characterization of ectomycorrhizal roots

Soil cores (15 cm^3) were dug from moist temperate oak forests close enough to *Quercus floribunda* tree trunks to ensure that they contained *Quercus* roots. Twenty to twenty-five soil cores were taken from every sampling site and stored in polythene bags to prevent evaporation. In the laboratory, each soil core was drenched in water up to 1–2 hours and then held over a 2 mm sieve beneath running water to detach roots from soil particles. Ectomycorrhizal roots were sorted into morphotypes using a Meiji Techno EMZ-5TR stereomicroscope. The morphotypes were then kept in distilled water in McCartney bottles, with replicates reserved in 2% CTAB buffer at 8°C for molecular analysis. The material was deposited in Lahore Herbarium, Department of Botany, University of the Punjab, Quaid-e-Azam Campus, Lahore, Pakistan (LAH).

Morphological analyses of basidiomata

For microscopic studies, tissues of pileus, gills, annulus, stipe, and volva were mounted in 5% KOH, stained with 1% aqueous Congo red (w/v), and observed under a Labomed light microscope. Basidiospore dimensions include length and width ranges (with parenthetical extreme values), range of Q coefficients (L/W), mean length and width ($L_m \times W_m$), and mean Q value (Q_m). The collection was vouchered and deposited in Lahore Herbarium, Department of Botany, University of the Punjab, Quaid-e-Azam Campus, Lahore, Pakistan (LAH).

DNA extraction, amplification, and sequencing

DNA was extracted using a modified CTAB method (Bruns 1995). The nuclear ribosomal internal transcribed spacer (ITS) locus was amplified using the fungal-specific ITS1F primer (5-CTTGGTCATTTAGAGGAAGTAA-3; Gardes & Bruns 1993) and the eukaryotic ITS4 primer (5-TCCTCCGCTTATTGATATGC-3; White & al. 1990). The amplified products were sent to Macrogen Inc. in Korea for purification and sequencing.

For identification of ectomycorrhiza, DNA was extracted using the Qiagen DNeasy Plant Mini Kit, and the ITS was amplified using the ITS1F/ITS4 primers. Amplicons obtained from ectomycorrhizal roots were purified with Exonuclease I and Shrimp Alkaline Phosphatase enzymes (Werle & al. 1994) and sequenced at the University of Florida Interdisciplinary Center for Biotechnology Research (www.biotech.ufl.edu/). Sequences were BLAST searched at NCBI (blast.ncbi.nlm.nih.gov/Blast.cgi), and closely related *Amanita* sequences were retrieved from GenBank. All the sequences were assembled in BioEdit (Hall 1999) and trimmed to facilitate alignment. A maximum likelihood tree was inferred for the ITS alignment using RAxML-HPC2 v 8.1.11 (Stamatakis 2014) with a GTRCAT nucleotide substitution model. Rapid bootstrapping was performed with 1000 bootstrap iterations. All phylogenetic analyses were performed on the CIPRES Science Gateway v3.1 (Miller & al. 2010). The phylogeny from ML analysis was displayed with FigTree v1.4.2 (tree.bio.ed.ac.uk/software/figtree/) and then edited in Adobe Illustrator. All newly generated sequences were submitted to GenBank.

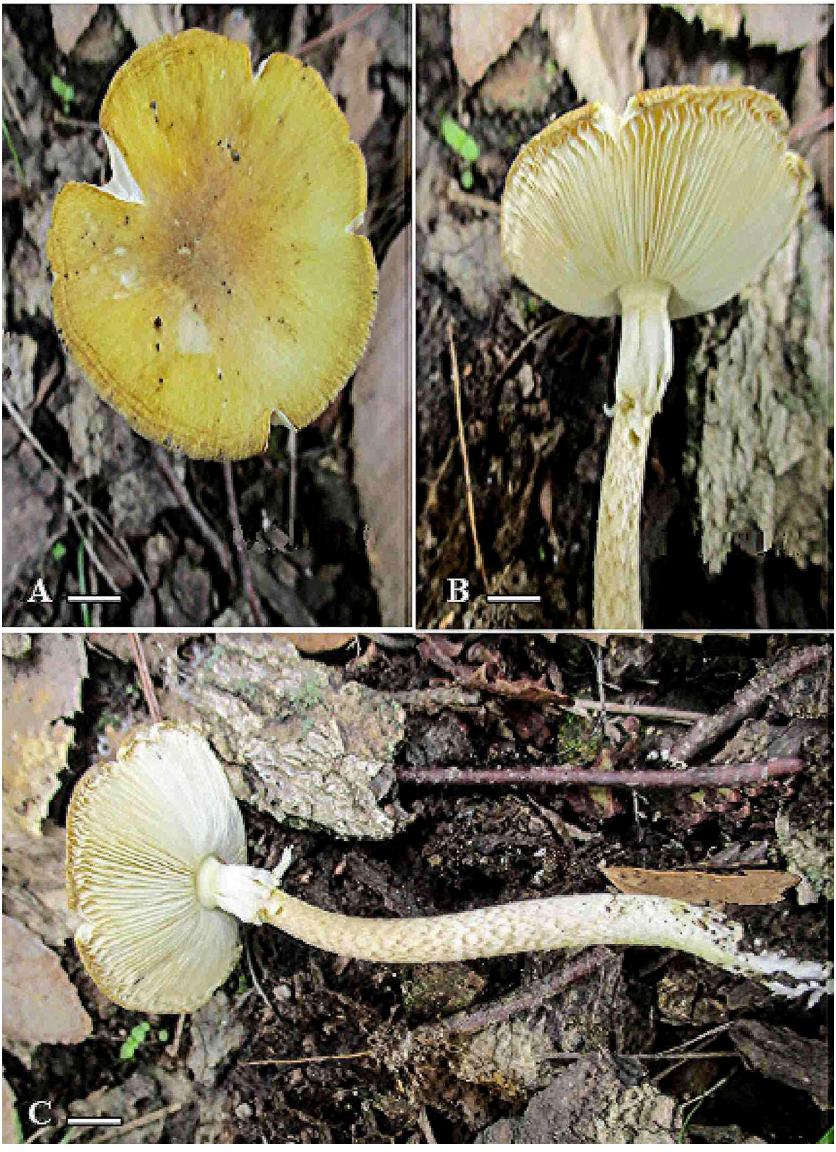

Fig. 1. *Amanita subjunquillea* (LAH35854).
Basidiomata. Scale bars: A = 0.79 cm; B = 1.17 cm; C = 1.02 cm.

Taxonomy

Amanita subjunquillea S. Imai, Bot. Mag., Tokyo 47: 424 (1933) Figs 1, 2

Pileus 60–70 mm diam, plano-convex with a slightly depressed disc and slightly uplifted margin, dark orange (10YR9/2) at disc, becoming light brown (5Y 9/4) toward margin; context white (5Y9/2) when cut. Lamellae free, close, entire, margins whitish (10Y9/2) to pale yellow (10Y 9/4). Lamellulae truncated, abundant, irregularly distributed. Stipe 120–130 × 5–15 mm, cylindrical, slightly tapering upwards, whitish (5Y9/2) at the base and pale yellowish (5Y9/4) toward the apex, covered by pale yellowish (5Y9/4) fibrillose squamules. Annulus superior, membranous, persistent, pendant, whitish (5Y 9/2) to pale yellow (5Y 9/4). Volva limbate, membranous, white.

Basidiospores (3.8–)4.3–5.7(–6.1) × (3.8–)4.1–5.2(–5.6) µm, Q = 1–1.2(–1.3), $L_m \times W_m = 5.1 \times 4.6$ µm, $Q_m = 1.1$, globose to subglobose, hyaline in 5% KOH, amyloid in Melzer's reagent, thin-walled, smooth, monoguttulate; apiculus 0.6–1 µm long. Basidia 20–27 × 8–9 µm, 2–4-spored, thin-walled, hyaline, narrowly clavate to clavate; sterigmata 1–2 µm long. Pileipellis hyphae 4–6 µm diam, branched, filamentous, septate, ellipsoid to subcylindrical, hyaline. Volva cells globose, ovoid to broadly lageniform 7–35 µm diam, 19–68 µm long; filamentous hyphae 3.1–4.5 µm diam. Stipitipellis hyphae 12–16 µm diam, septate, unbranched, thin-walled, hyaline in 5% KOH. Partial veil elements 34–55 × 10–21 µm, ellipsoid to clavate intermixed with short septate filaments 1–2 µm diam. Clamp connections absent throughout.

Material examined: **PAKISTAN. Khyber Pakhtunkhwa Province**, **Buner District**, Gokand Valley, Bottle Green Mountains, 3183 m a.s.l., under *Quercus floribunda* [= "*Q. dilatata*" Royle], solitary, 13 August 2017, Muhammad Ishaq IB124 (LAH-35854; GenBank MH620472).

Habitat: Solitary on soil under *Quercus floribunda.*

Ectomycorrhizal morphology Fig. 3

Mycorrhizal system monopodial pinnate, 20–45 mm, 0.7–1 mm thick, profusely branched, surface smooth, light brown (5YR8/4), older parts dark reddish brown (5YR3/4). Unramified ends slightly straight to (infrequently) bent, cylindrical. Mantle brown (5YR8/5), not translucent. Outer mantle hyphae 2.7–3.4 µm diam, thin-walled, septate, branched, cells similar in size to inner mantle hyphae, sub-cylindrical to ellipsoid, end cells obtuse, no clamp connections. Inner mantle hyphae 2.4–3.5 µm diam, thin-walled, septate,

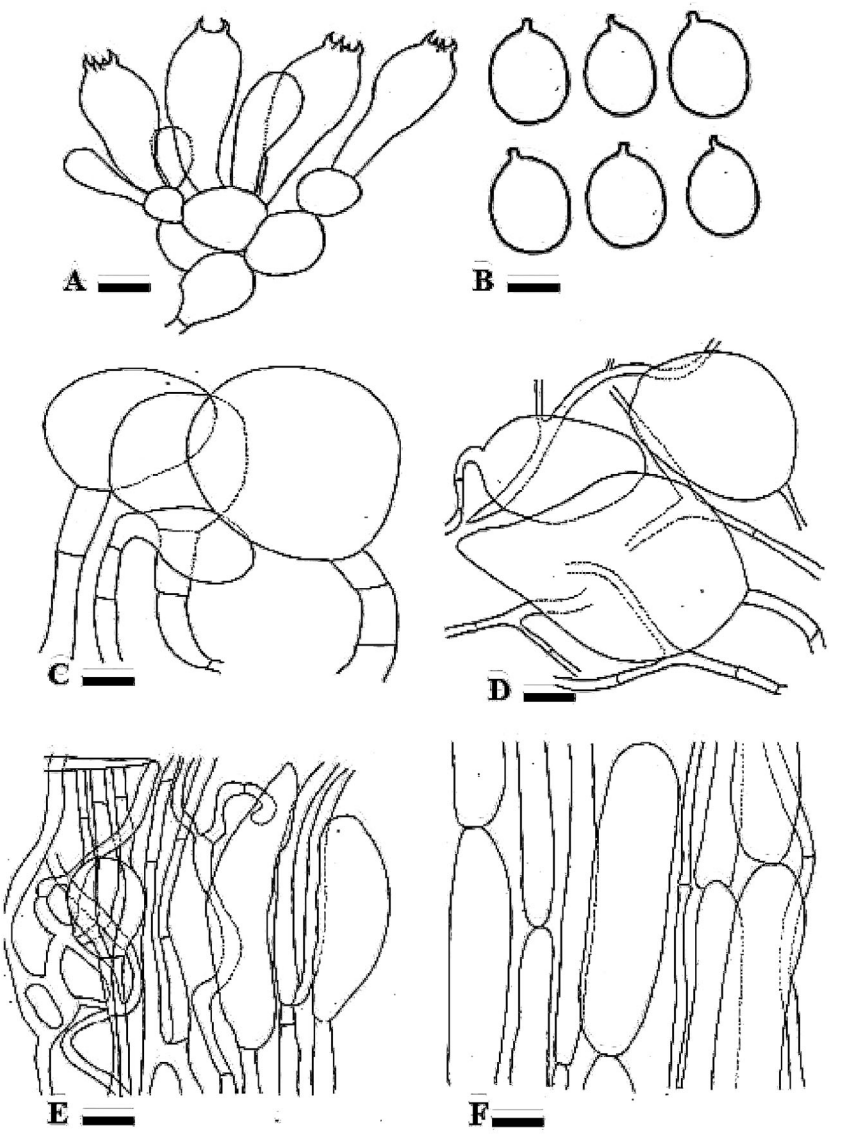

Fig. 2. *Amanita subjunquillea* (LAH35854). A. Basidia; B. Basidiospore; C. Partial veil elements; D. Volva; E. Pileipellis; F. Stipitipellis. Scale bars: A = 7.5 µm; B = 3 µm; C, D = 7 µm; E = 4.5 µm; F = 1 µm.

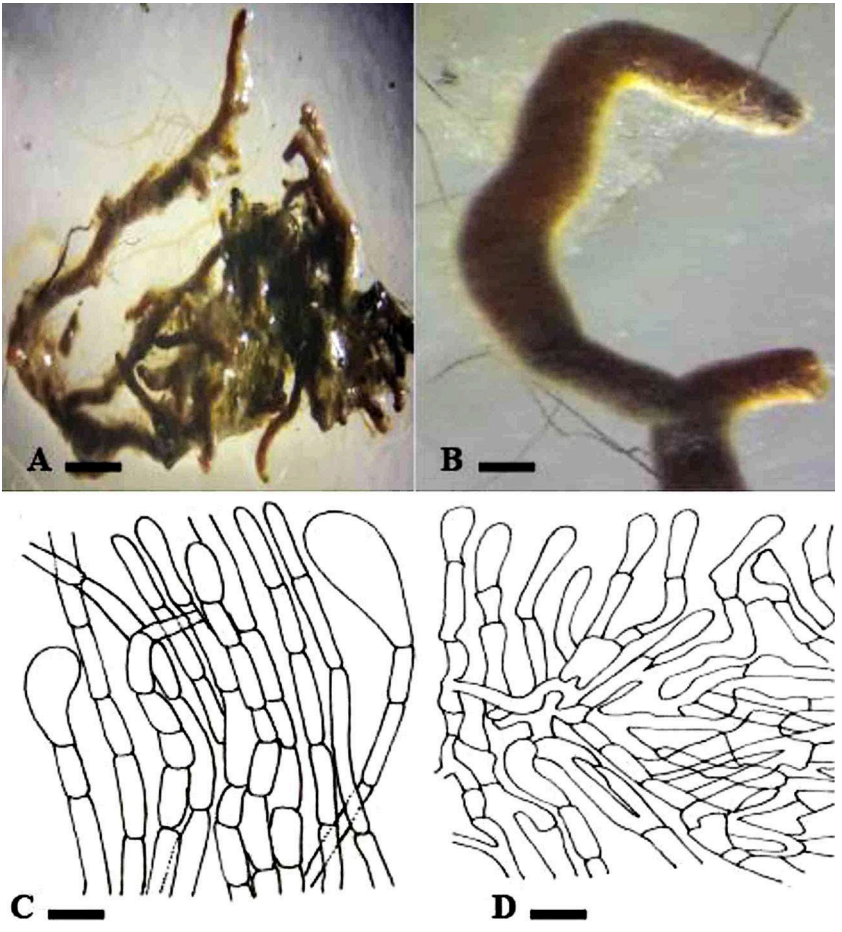

Fig. 3. *Amanita subjunquillea/Quercus floribunda* mycorrhizae (LAH-EM101, LAH-EM102). A., B. Ectomycorrhizal roots; C. Inner mantle layer cells; D. Outer mantle layer cells. Scale bars: A = 1.91 mm; B = 0.54 mm; C = 4.5 μm; D = 10.2μm.

unbranched, narrowly clavate to clavate, 6–11 μm long, end cells obtuse, cystidia infrequent. Emanating hyphae numerous, white. Rhizomorphs white (2.5R9/2), thick, frequent, abundant.

Material examined: **PAKISTAN. Khyber Pakhtunkhwa Province, Shangla/ Swat boundary**, Toa, 2100 m a.s.l, associated with roots of *Quercus floribunda*, 15

July 2015, Arooj Naseer & Abdul Nasir Khalid ANT210 (LAH-EM101; GenBank MH998629); ANT211 (LAH-EM102; GenBank MH998628); ANT212 (LAH-EM103; GenBank, MH998627).

Phylogenetic results **Fig. 4**

The aligned ITS dataset, including sequences retrieved from GenBank, comprised 30 nucleotide sequences. Of the 665 characters in the final dataset, 275 were conserved, 363 were variable and parsimony-uninformative, and 228 were parsimony-informative. Our ML tree contains three sections: *Amanita* sect. *Validae, A.* sect. *Amanita*, and *A.* sect. *Phalloideae*. The

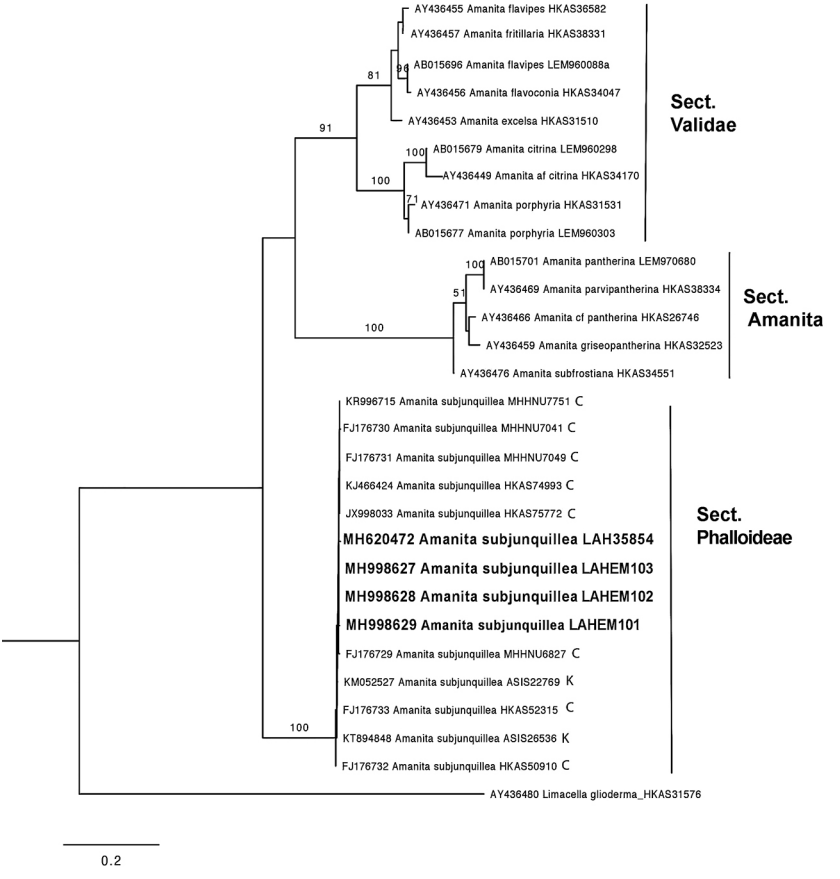

Fig. 4. Molecular phylogenetic reconstruction of *Amanita* spp. based on ITS sequences. The best scoring ML tree (–lnL = 1591.4482) is shown. Only bootstrap values >50 are shown. *Amanita subjunquillea* sequences from China are annotated as [C] and those from Korea as [K].

sequences from our Pakistani specimen and ectomycorrhizal roots, grouped within *A.* sect. *Phalloideae* with previously generated sequences of *A. subjunquillea* from China and South Korea.

Comparison of the ITS sequences revealed 19 polymorphic positions among different collections of *A. subjunquillea* from Pakistan, China, and Korea. The Pakistani sequence contains eleven polymorphic positions (at positions 2, 7, 32, 35, 107, 204, 504, 507, 553, 648, and 667), with most nucleotides at these allelic positions resembling Chinese samples. However, one Chinese sample is closer to the Korean specimen KT894848. Maximum nucleotide differences were observed in Korean sample KM052527, with seven positions differing from all other collections, including Korean sample KT894848. It is possible that the KM052527 ITS sequence needed additional purification, but more sequences are required to verify these seven nucleotide differences.

Discussion

Amanita subjunquillea is a deadly poisonous mushroom distinguished by a brownish yellow to mustard yellow pileus, non-appendiculate margins, and a subglobose to subclavate to turnip-shaped basal bulb (Yang 1997, 2005; Cai & al. 2016; Cui & al. 2018).

Macroscopic and microscopic details of the examined specimens match the protologue description of *A. subjunquillea* (Imai 1933), except for pileus coloration patterns. The pileus color of the Pakistani specimen is dark orange at the disc and pale yellow toward the margin, while the type specimen is described with the pileus darker at the center and brownish yellow, dirty citrine yellow, or mustard yellow toward the margin. This is the only macroscopic difference separating our collection from the type; the nearly cylindrical stipe that tapers slightly upward and is covered with yellowish fibrillose scales matches the type description. The spore and basidial shapes are similar among all collections, but the basidia and spore sizes are smaller in our specimen compared with Indian and East Asian specimens. Clamp connections are absent in all specimens.

Our phylogenetic analysis clustered ITS sequences from our Pakistani collection and ectomycorrhizal roots in *A.* sect. *Phalloideae* with ten other sequences belonging to *A. subjunquillea.* We found some variation among the Chinese, Korean, and Pakistani nucleotides; nucleotide differences within same species from different localities of China were also observed by Zhang & al. (2010). The type collection was sampled under warm temperate

conditions in Tokyo whereas our sampling site lies in a moist temperate region. These environmental differences can affect gene function so that same species collected from different geographic regions may show minor variation in both morphological and molecular characters.

Amanita subjunquillea is endemic to East Asia (Zhang & al. 2010). This is the first published report of its ectomycorrhizal association with *Quercus floribunda* based on morphological, anatomical, and phylogenetic data. *Amanita subjunquillea* has previously been reported from Japan, Korea, India, and China, to which we now add Pakistan.

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Literature cited

Bas C. 1969. Morphology and subdivision of *Amanita* and a monograph of its section *Lepidella*. Persoonia 5(4): 285–573.

Bas C. 2000. A broader view on *Amanita*. Bollettino del Gruppo Micologico G. Bresadola 43: 9–12.

Bhatt RP, Tulloss RE, Semwal KC, Bhatt VK, Moncalvo JM, Stephenson SL. 2003. *Amanitaceae* reported from India. A critically annotated checklist. Mycotaxon 88: 249–270.

Bhatt RP, Semwal KC, Upadhyay RC. 2007.New records of section *Phalloideae* of the genus *Amanita* from Garhwal Himalaya, India. Mushroom Research 16(2): 61–67.

Bruns TD. 1995. Thoughts on the processes that maintain local species diversity of ectomycorrhizal fungi. Plant and Soil 170(1): 63–73. https://doi.org/10.1007/BF02183055

Cai Q, Cui YY, Yang L. 2016. Lethal *Amanita* species in China. Mycologia 108(5): 993–1009. https://doi.org/10.3852/16-008

Cui YY, Cai Q, Tang LP, Liu JW, Yang ZL. 2018. The family *Amanitaceae*: molecular phylogeny, higher–rank taxonomy and the species in China. Fungal Diversity 91(1): 5–230. https://doi.org/10.1007/s13225-018-0405-9

Cho HJ, Park MS, Lee H, Oh SY, Jang Y, Fong JJ, Lim YW. 2015. Four new species of *Amanita* in Inje County, Korea. Mycobiology 43(4): 408–414. https://doi.org/10.5941/MYCO.2015.43.4.408

Corner EJH, Bas C. 1962. The genus *Amanita* in Singapore and Malaya. Persoonia 2: 241–304. www.repository.naturalis.nl/record/532339

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes application to the identification of mycorrhizae and rusts. Molecular Ecology 2(2): 113–118. https://doi.org/10.1111/j.1365-294X.1993.tb00005.x

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41(41): 95–98.

Jabeen S, Kiran M, Khan J, Ahmad I, Ahmad H, Sher H, Khalid AN. 2019. *Amanita ahmadii*, a new species of *Amanita* subgenus *Amanitina* section *Validae* from Pakistan. MycoKeys 56: 81–99. https://doi.org/10.3897/mycokeys.56.31819

Khan A, Gilani SS, Hussain F, Durrani MJ. 2003. Ethnobotany of Gokand valley, District Buner, Pakistan. Journal of Biological Sciences 6: 363–369.

Kim KH, Choi SU, Park KM, Seok SJ, Lee KR. 2008. Cytotoxic constituents of *Amanita subjunquillea.* Archives of Pharmacal Research 31(5): 579–586. https://doi.org/10.1007/s12272-001-1196-3

Kiran M, Khan J, Sher H, Pfister DH, Khalid AN. 2018. *Amanita griseofusca*: A new species of *Amanita* in section *Vaginatae* from Malam Jabba, Swat, Pakistan. Phytotaxa 364(2): 181–192. https://doi.org/10.11646/phytotaxa.364.2.5

Kirk PM, Cannon PF, Minter DW, Stalpers JA. 2008. Ainsworth Bisby's dictionary of fungi. 10th ed. CABI, Wallingford, UK. https://doi.org/10.1079/9780851998268.0000

Li HJ, Xie JW, Zhang S, Zhou YJ, Ma PB, Zhou J, Sun CY. 2015. *Amanita subpallidorosea*, a new lethal fungus from China. Mycological Progress 14:43 [11 p.]. https://doi.org/10.1007/s11557-015-1055-x

Miller MA, Pfeiffer W, Schwartz T. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. [8 p.], in: Gateway Computing Environments Workshop, 14 Nov. 2010. New Orleans, LA. https://doi.org/10.1109/GCE.2010.5676129

Munsell Soil Color Charts. 1975. Munsell Color Co, Baltimore, MD.

Rho HJ, Kim JH, Kang HR, Lee MK, Hyu SH, Kang YM, Kim NS. 2000. Clinical manifestations of *Amanita subjunquillea* poisoning. Korean Journal of Medicine 58(4): 453–461.

Saba M, Haelewaters D, Fiaz M, Khalid AN, Pfister DH. 2019. *Amanita mansehraensis*, a new species in section *Vaginatae* from Pakistan. Phytotaxa 409(4): 189–201. https://doi.org/10.11646/phytotaxa.409.4.1

Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post–analysis of large phylogenies. Bioinformatics 30(9): 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Thongbai B, Tulloss RE, Miller SL, Hyde KD, Chen J, Zhao R, Raspé O. 2016. A new species and four new records of *Amanita* (*Amanitaceae*; *Basidiomycota*) from Northern Thailand. Phytotaxa 286(4): 211–231. http://dx.doi.org/10.11646/phytotaxa.286.4.1

Tulloss RE. 2005. *Amanita*—distribution in the Americas, with comparison to eastern and southern Asia and notes on spore character variation with latitude and ecology. Mycotaxon 93: 189–231.

Yang ZL. 1997. Die *Amanita*-Arten von Südwestchina. Bibliotheca Mycologica 170. 240 p.

Yang ZL. 2005. Flora fungorum sinicorum, vol. 27. *Amanitaceae*. Science, Beijing [in Chinese].

Yang ZL, Cai Q, Cui YY. 2018. Phylogeny, diversity and morphological evolution of *Amanitaceae*. Biosystematics and Ecology Series 34: 359–380.

Zhang LF, Yang JB, Yang ZL. 2004. Molecular phylogeny of eastern Asian species of *Amanita* (*Agaricales*, *Basidiomycota*): taxonomic and biogeographic implications. Fungal Diversity 17: 219–238.

Zhang P, Chen ZH, Xiao B, Tolgor B, Bao HY, Yang ZL. 2010. Lethal amanitas of East Asia characterized by morphological and molecular data. Fungal Diversity 42: 119–133. https://doi.org/10.1007/s13225-010-0018-4

Zhang P, Tang LP, Cai Q, Xu JP. 2015. A review on the diversity, phylogeography and population genetics of *Amanita* mushrooms. Mycology 6(2): 86–93. https://doi.org/10.1080/21501203.2015.1042536